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Enhanced cytocompatibility and functional group content of poly(L-lysine) dendrimers by grafting with poly(oxazolines)†

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When considering the design of an advanced drug delivery system, a common desirable attribute is to have a prolonged residence time in blood circulation so that accumulation and localised payload release may occur at the site of interest (*e.g.* a tumour). Polyethylene glycol (PEG) has been a gold standard for fulfilling this requirement, and consequently has been well investigated as a material for surface modification of dendrimers. As an alternative, we have explored the use of polyoxazolines (POZ)s as materials for modifying the surface of a generation 5 L-lysine dendrimer and found that there was a significant improvement in the biocompatibility properties over the unmodified dendrimer. One particularly useful advantage of POZ over PEG lies in the main-chain pendant groups available that we were able to exploit to impart functionality. Modifying the POZ to have pendant carboxyl groups led to a novel modified dendrimer with significantly more sites for conjugation. With this, we have demonstrated a sixfold increase in the loading of coumarin (our model compound) when compared to a non-functional POZ equivalent.

Introduction

Dendrimer-drug conjugates, typically in the range of 2–25 nm in size, are increasingly popular as drug delivery systems because they are water soluble, have near-monomodal molecular weight distributions, reproducible synthesis steps and have large numbers of surface groups available for drug conjugation. In addition, these accessible functional groups can be easily modified for property enhancement, for example by substitution with PEG chains to reduce cytotoxicity and systemic clearance. PEGylation has been widely employed with polyamidoamine (PAMAM)[1–4] and poly(L-lysine) dendrimers.[5–8] The circulation time of these cationic dendrimers is significantly improved when modified with PEG as the surface charge is neutralised and PEG tends to have low reticuloendothelial system uptake and low immunogenicity. These types of drug delivery systems can also benefit from accumulation at the site of interest (*e.g.* a tumour *via* the Enhanced Permeation and Retention (EPR) effect) allowing for desirable localised drug delivery.[9] However, the relative inertness of PEG can also be disadvantageous, as it is difficult to functionalise PEG itself with components like targeting ligands. To improve the diversity and functionality of these derivatised poly(amine) dendrimer systems we chose to use POZs as an alternative material to modify the dendrimer surface. POZs have become popular alternatives to PEG, in-part owing to the diversity offered by the pendant group that could in turn be derivatised to a wide range of different functionalities with many different resulting polymer properties.[10–12] Furthermore, the implementation of microwave synthesis for POZs has significantly improved reaction rates whilst maintaining living polymerisation, leading to well-defined polymers with facile introduction of chain-end functionality.[13–15] POZs have been reported to have similar stealth and biocompatibility properties to PEG[16–18] and thus have been widely used in the field of investigational polymer therapeutics.[19–22] With this in mind we considered two of the most widely used types of POZ for bioapplications; poly(2-methyl-2-oxazoline) (PMOx) and poly(2-ethyl-2-oxazoline) (PEOx) for the surface modification of a generation 5 L-lysine dendrimer. The attraction of this system lies in the fine-tuning of the properties that could be achieved by using POZ through main-chain and end group variation *e.g.* improved water solubility and improved viscosity properties. In this manuscript, the most important main-chain variation that we have explored is a POZ with a pendant ester group that was synthesized and used to modify pre-formed lysine dendrimers. Subsequent deprotection of the ester groups yielded carboxyl functionalities

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along the backbone and therefore significantly more sites for drug conjugation than currently available using existing PEG and PMOx/PEOx modified dendrimers. Without these additional conjugation sites these types of dendrimers are limited to the remaining (lysine) functional groups from the dendrimer scaffold. These novel dendrimer types have been explored for properties which would underlie their potential application as drug delivery systems using a model compound (coumarin) to conjugate to the dendrimers as a proof-of-principle experiment to test drug loading and cytocompatibility.

Materials and methods

Materials

All reagents and solvents were purchased from Sigma Aldrich with exception of MeO-PEG-COOH (2000 Da) (Iris Biotech GMBH), Boc-L-Lys(Boc)-ONp & 2-chloro-4,6-dimethoxy-1,3,5-triazine (Chem-Impex International), 2-chloroethylamine hydrochloride (Fluorochem Inc.), sodium methoxide 5.4 M (Acros Organics), Fetal calf serum (PAA), NucView™ (Biotium). Sartorius AG VivaFlow® 50 cassettes (30 kDa MWCO) were purchased from VWR. Polyethylene oxide standards were purchased from Agilent Technologies as a kit. Wistar rat heparinized whole blood was ordered fresh from Harlan Laboratories UK Ltd and used the same day.

Equipment

^{1}H NMR analysis was performed on a Bruker AV400. Gel Permeation Chromatography-Refractive Index (GPC)-RI was performed on a Malvern TDA302 with either an Agilent Technologies aquagel-OH-20 column running 50/50 MeOH/40 : 10 mM $NaNO_3$: NaH_2PO_4 as the eluent or a Tosoh Bioscience TSKgel GMPWxl column with 40 : 10 mM $NaNO_3$: NaH_2PO_4 as the eluent. The system calibration was performed using a single poly(ethylene oxide) standard (16 100 g mol^{-1}) and absolute molecular weights determined using the refractive index and light scattering signals. Organic GPC was performed with DMF (0.05 M LiBr) and a universal calibration using PEG/PEO narrow standards ranging from 194 g mol^{-1} to 16 100 g mol^{-1}. GPC-UV was performed on a Waters HP1100 fitted with a Tosoh Bioscience TSKgel GMPWxl column with PBS as the eluent and UV detection at 220 nm. Haemoglobin levels were recorded using a HemoCue® Hb210 and HemoCue plasma/low Hb system for high and low hemoglobin concentration measurements. Samples were centrifuged in a Eppendorf™ MiniSpin™ microcentrifuge. Dynamic light scattering was performed on a Malvern Zetasizer® at a temperature of 25 °C. Samples were made to concentrations of 5 mg mL^{-1} in 10 mMolar NaCl solution or a 40 : 10 mMolar ratio of $NaNO_3$: NaH_2PO_4 at pH 7.4 and were filtered using a 0.2 μm syringe filter prior to measurement. Data was obtained using Malvern Zetasizer software version 6.21J. Haemoaggregation was measured on a Zeiss Axio Scope. A1 at 40× magnification. Images were taken with an integrated Zeiss Axio Cam ERc 5 s camera and manipulated with Zeiss ZEN Lite 2011 software.

Methods

4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride and tetrafluoroborate. DMTMM·Cl[23] and DMTMM·BF_4 [24] were synthesised according to methods reported previously.

***N*-(2-Aminoethyl)-2-oxo-2*H*-chromene-3-carboxamide (coumarin-NH_2).** Coumarin-3-carboxylic acid (200 mg, 1.05 mmol) and DMTMM·BF_4 (518 mg, 1.58 mmol) were weighed into a reaction vial. DMF (5 mL) was added, followed by *tert*-butyl (2-aminoethyl)carbamate (253 mg, 1.58 mmol) dissolved in anhydrous DMF (0.5 mL). The reaction was followed to completion by UPLC-MS (approximately 30 min). The reaction mixture was diluted with EtOAc (20 mL), and washed with saturated $NaHCO_3$ (2 × 10 mL) and saturated brine (2 × 10 mL). The organic layer was dried over $MgSO_4$, filtered and evaporated to afford crude product, which was purified by flash silica chromatography, elution gradient 20 to 70% 3 : 1 EtOAc/EtOH in heptane to give *tert*-butyl (2-(2-oxo-2*H*-chromene-3-carboxamido)ethyl)carbamate (264 mg, 76%). The boc-deprotecting group was removed by stirring the solid in DCM (7.5 mL) and TFA (1 mL, 16 eq.) for 4 h and removing the solvents under vacuum at room temperature overnight to give the title compound. Yield = 275 mg, 100% (TFA salt). MS ES$^+$ = 333.3 (analytical ESI S2 Fig. 1–4†).

Generation 5 L-lysine dendrimer. The dendrimer was synthesised to generation 2 according to a literature method.[25] Successive generations were synthesised from repetitive steps involving the deprotection of the previous generation's lysine boc-protecting groups using trifluoroacetic acid (50 eq. to boc groups) in DCM (10× volume) followed by partial evaporation and precipitation into 20× excess of diethyl ether. A white solid was obtained through filtration and overnight drying in a vacuum oven. Further reaction with Boc-L-Lys(Boc)-Onp in the presence of triethylamine produced the next generation boc-protected dendrimer. Reaction times, equivalents of reagents and yields are summed up in ESI S1 Table 1.† ^{1}H NMR and MALDI-TOF-MS ESI S2 Fig. 5.†

HOOC-poly(2-methyl-2-oxazoline)-OMe (1a). Freshly distilled 2-methyl-2-oxazoline (2 g, 23.5 mmol) (over CaH_2, N_2) was added to an oven-dried, nitrogen-purged microwave tube fitted with a septum. Anhydrous acetonitrile (4 mL) was added *via* syringe, followed by *tert*-butyl bromoacetate (0.195 g, 1 mmol). The reaction mixture was transferred to a microwave reactor and heated to 140 °C for 30 min. The solution was cooled to 0 °C using an ice bath and then sodium methoxide solution (2 M, 2.5 mL, 5 eq.) was added dropwise and then stirred for 10 minutes at 0 °C. Sodium hydroxide solution (2 M, 2 mL) was added and the reaction mixture was stirred overnight at rt. The reaction mixture was acidified to pH 5 using HCl (2 M) and then the solvents were removed by rotary evaporation. Anhydrous ethanol (dried over 3A molecular sieves) (15 mL) was added and the flask agitated under a nitrogen blanket until the polymer had dissolved leaving behind the NaCl salts which were filtered off. The ethanol was evaporated and the process repeated. The polymer was dissolved in a minimum

volume of anhydrous ethanol and was added to a large volume of diethyl ether (400 mL), filtered and dried under vacuum. To remove traces of ethanol the polymer was freeze-dried to obtain the title compound as a white solid. Yield = 1.9 g, 89%. ^{1}H NMR ESI S2 Fig. 6.†

HOOC-poly(2-ethyl-2-oxazoline)-OMe (1b). This compound was synthesised following the method for **1a**, but in place of anhydrous ethanol, anhydrous DCM was used to remove the unwanted NaCl salts. The polymer was obtained as a white solid after precipitation into diethyl ether, filtering and drying overnight under vacuum at 40 °C. Yield = 1.74 g, 83%. ^{1}H NMR ESI S2 Fig. 7.†

MeO-PEG-COOH (2000 Da) (1c). This polymer was purchased from Iris Biotech GMBH. ^{1}H NMR ESI S2 Fig. 8.†

Methyl 4-(2-chloroethyl)amino)-4-oxobutanoate. This compound was synthesised according to the literature[26] using methyl 4-chloro-4-oxobutyrate (8 g, 53.13 mmol) to give the title compound (6.92 g, 67.3%) as a yellow oil. The crude product was used without further purification.

Methyl 3-(4,5-dihydrooxazol-2-yl)propanoate. Methyl 4-((2-chloroethyl)amino)-4-oxobutanoate (6.9 g, 35.64 mmol) and anhydrous sodium carbonate (2.83 g, 26.73 mmol) were mixed under vacuum at 0.5 mbar and gradually heated to 157 °C in a Kugelrohr apparatus. The title compound distilled off as a colourless oil and was collected and used immediately. Yield = 2.70 g, 48.2%.

HOOC-poly(methyl-4-oxobutanoate-2-oxazoline)-ethyl-2-thioethanoate (HOOC-PEsterOx-COOMe) (1d). Methyl 3-(4,5-dihydrooxazol-2-yl)propanoate (2.7 g, 17.18 mmol) was added to an oven-dried, nitrogen-purged microwave tube fitted with a stirrer bar and septum. Anhydrous acetonitrile (5 mL) was added, followed by *tert*-butyl bromoacetate (0.181 mL, 1.23 mmol). The reaction mixture was transferred to a Biotage microwave and heated to 140 °C for 30 min. After cooling to rt, ethyl thioglycolate (2 eq.) was added followed by anhydrous triethylamine (2 eq.) and stirred overnight. The reaction mixture was added dropwise to rapidly stirring diethyl ether (250 mL) to form a white precipitate that was filtered and dried under vacuum. The solid was then taken up in DCM (100 mL) and washed with saturated $NaHCO_3$ (3 × 30 mL) before washing with saturated brine (2 × 30 mL). The organic layer was dried over magnesium sulphate, filtered and the solvents evaporated. The film was taken up in DCM (25 mL) and TFA (4 mL) was added dropwise and stirred for 5 h. The solvents were removed under vacuum and the film was taken up in deionised water (50 mL) before freeze-drying to give the title compound as a white solid. Yield = 2.8 g, 90% ^{1}H NMR ESI S2 Fig. 9.†

G5-PLL[PMOx-OMe]$_x$[NH$_2$]$_y$ (2a). The generation 5 L-lysine dendrimer (100 mg, 6.53 μmol) and **1a** (667 mg, 0.31 mmol, 48 eq.) were dissolved together in deionised water (5 mL). DMTMM·Cl (174 mg, 0.63 mmol, 96 eq.) was added as a solid and after dissolution the pH of the solution was adjusted to ~8 using saturated $NaHCO_3$ solution. The reaction mixture was stirred at rt for **4d**. The pH of the reaction was monitored and maintained at 8. Afterwards, the reaction mixture was acidified to pH 5 using HCl (2 M) and purified using a VivaFlow® cassette in deionised water (30 kDa MWCO) connected to a peristaltic pump. Removal of unreacted POZ and DMTMM·Cl was monitored by SEC-UV (220 nm) and the reaction was typically free of low molecular weight impurities after 1–2 hours. The title compound was obtained as a white solid after freeze-drying. Yield = 348 mg, 70%. (Yield calculated according to a desired 50% modified polymer.)

G5-PLL[PEOx-OMe]$_x$[NH2]$_y$ (2b). The method above was used but with **1b** in place of **1a**. Yield = 343 mg, 69% ^{1}H NMR ESI S2 Fig. 10.†

G5-PLL[PEG-OMe]$_x$[NH2]$_y$ (2c). The method for **2a** was used but with commercially available HOOC-PEG-OMe (**1c**). Yield = 373 mg, 79% ^{1}H NMR ESI S2 Fig. 11.†

G5-PLL[P(2-ester-2-oxazoline)]$_x$[NH$_2$]$_y$ (2d). The generation 5 L-lysine dendrimer (100 mg, 6.53 μmol) and **1d** (697 mg, 0.31 mmol, 48 eq.) were dissolved together in anhydrous DMF (5 mL). DMTMM·BF_4 (206 mg, 0.63 mmol, 96 eq.) was added as a solid and after dissolution *N*-methyl morpholine (~20 μL) was added. The reaction was then stirred for 48 h at rt. The reaction mixture was diluted with ethyl acetate (3 mL) and the solution added to rapidly stirring diethyl ether (250 mL) to form a white precipitate. The precipitate was filtered, dried under vacuum and then taken up in deionised water (50 mL). The pH was adjusted to 5 with HCl (2 M) and the polymer purified similarly to **2a**. The title compound was obtained as a white solid. Yield = 425 mg, 82%. ^{1}H NMR ESI S2 Fig. 12.†

G5-PLL[PMOx-OMe]$_x$[COOH]$_y$ (3a) and G5-PLL[P(2-ester-2-oxazoline)]$_x$[COOH]$_y$ (3d). The residual amine groups on **2a** and **2d** were modified to acids using succinic anhydride. Briefly, the dendrimer (200 mg, 0.08 mmol (NH_2)) was dissolved in anhydrous methanol (4 mL). Succinic anhydride (50.1 mg, 0.50 mmol, 6 eq. to NH_2) was added as a solid, followed by triethylamine (23 μL, 0.17 mmol, 64 eq.). The reaction mixture was stirred at rt for 5 h before diluting with deionised water (40 mL) and acidifying to pH 5 with HCl (0.1 M) and purifying using VivaFlow® (30 kDa MWCO) for 1 hour. Both polymers were obtained as white solids after freeze-drying. Yield **3a** = 174 mg, 74%. **3d** = 195 mg, 94%. ^{1}H NMR ESI S2 Fig. 13† (**3a**).

G5-PLL[P(2-acid-2-oxazoline)]$_x$[COOH]$_y$ (4d). Polymer **3d** (150 mg, 1.70 mmol (ester repeat unit)) was dissolved in methanol (3 mL). LiOH (42 mg, 2 eq. to each repeat unit) dissolved in deionised water (1 mL) was added dropwise and the reaction mixture was stirred overnight at rt. The pH was adjusted to 8 before removing the solvent under vacuum. The solid was taken up in deionised water (20 mL) and the pH adjusted to 4–5 to form a white precipitate that became a gum on stirring. The water was decanted to waste and the gum washed with deionised water (20 mL) before freeze-drying the gum to obtain the title compound as a white solid. Yield = 111 mg, 89%. ^{1}H NMR ESI S2 Fig. 14.†

G5-PLL[PMOx-OMe]$_x$[coumarin]$_y$ (4a). Polymer **3a** (50 mg, 0.62 μmol) was dissolved in anhydrous DMF (2 mL) in a nitrogen purged reaction tube fitted with a stirrer bar and septum. DMTMM·BF_4 (9.8 mg, 0.03 mmol) was added and the solution stirred for 5 min. A preprepared solution of coumarin-NH_2

(8.62 mg, 0.02 mmol, 40 eq.) in anhydrous DMF (0.5 mL) was added *via* syringe. Triethylamine (3.5 μl, 0.02 mmol) in DMF (0.2 mL) was added and the reaction mixture was stirred at rt for 24 hours. The solution was then diluted with EtOAc (2 mL) and precipitated into diethyl ether (200 mL). The precipitate was collected, dried under vacuum and then dissolved in a 50/50 MeOH/water mixture (25 mL). The solution was passed through a 0.45 μm filter and then purified by VivaFlow® (30 kDa MWCO) gradually changing the eluent to pure deionised water. The title compound was obtained as a white solid after freeze-drying. Yield = 46 mg, 81%. ^{1}H NMR ESI S2 Fig. 15.†

G5-PLL[P(2-acid-2-oxazoline)]$_x$[coumarin]$_y$ (5d). This polymer was prepared in a similar method as for **5a**. Polymer **4c** 50 mg, 0.56 μmol was dissolved in anhydrous DMF (2 mL) in a nitrogen purged reaction tube fitted with stirrer bar and septum. DMTMM·BF_4 (35 mg, 0.11 mmol) was added and the solution stirred for 5 min. **Coumarin-NH$_2$** (34 mg, 0.10 mmol) was added as a solid directly and after dissolution, triethylamine (14 μl, 0.10 mmol) in DMF (0.2 mL) was added slowly drop-wise to avoid precipitation. The reaction mixture was stirred at rt for 24 h before diluting with EtOAc (2 mL) and precipitating the product by addition to rapidly stirring diethyl ether (200 mL). The precipitate was collected, dried under vacuum and then dissolved initially in a small volume of $NaHCO_3$ solution (2 mL) and then diluted in a 50/50 MeOH/water mixture (25 mL). The solution was passed through a 0.45 μm filter and then purified by VivaFlow® (30 kDa MWCO) gradually changing the eluent to pure deionised water. The title compound was obtained as a white solid after freeze-drying. Yield = 54 mg, 78%. ^{1}H NMR ESI S2 Fig. 16.†

Methodology for the cell viability and blood compatibility

Cell viability and proliferation assays. NIH 3T3 mouse fibroblasts were obtained from the Albert Wong Jefferson Cancer Institute. Cells were cultured at 37 °C/5% CO_2 in RPMI-1640, supplemented with 10% fetal calf serum and 2 mM glutamine, in the presence of penicillin–streptomycin. Cells were not used beyond passage number 10 for these experiments. Cells were seeded into 96-well plates at 5000 cells per well, in the presence of 1 μM NucView™ to enable the detection of apoptosis. Seeded cells were allowed to adhere overnight. In triplicate, the cells were treated with the modified dendrimers (except dendrimer **2d** which was not soluble enough to make a stock solution) & unmodified L-lysine dendrimer at 0.5 mg mL^{-1} (final concentration) in full media. Plates were incubated at 37 °C in an IncuCyte™ FLR (Essen BioScience) and were scanned every 4 hours for NucView™ fluorescence (apoptosis) and cell confluence. The apoptosis induced by each dendrimer was calculated relative to untreated controls and a maximum value obtained from the unmodified L-lysine dendrimer at 96 hours.

Blood compatibility studies. The blood compatibility studies were performed using Wistar rat blood collected in lithium heparinised tubes. 250 μl of lithium heparinised whole blood was placed in to a 1 ml Eppendorf tube and 250 μl of the sample (4 mg ml^{-1} to measure at 2 mg ml^{-1}) was added and mixed gently for 45 seconds. 500 μl of physiological saline was added to quench haemolysis and gently mixed. The tube was placed into a centrifuge and spun at 600*g* for 5 minutes. A sample of the supernatant was taken with a capillary stick, and placed at the top of a micro-cuvette (plasma/low Hb) allowing 20 μl to be automatically drawn up by capillary action. The cuvette was transferred to a Hemocue photometer (plasma/low photometer) and the haemolysis measurement (released haemoglobin, g dl^{-1}) was taken. To measure the haemoaggregation, 100 μL of lithium heparinised whole blood was diluted with 100 μL of sample in physiological saline (4 mg ml^{-1}) and gently mixed for 30 seconds. 20 μL of the mixture was transferred to a microscopy slide and a cover carefully placed on top. The red blood cells were inspected at 40× magnification and images were captured using the integrated Zeiss Axio camera.

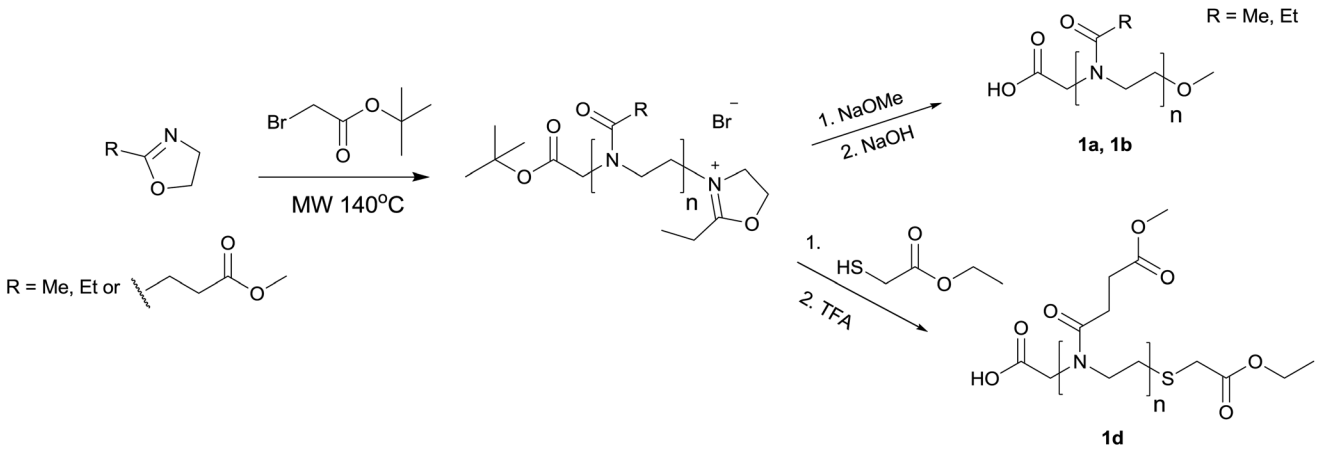
Results and discussion

PMOx, PEOx and PEsterOx synthesis

POZs **1a** and **1b** (Scheme 1) were synthesised to have similar chain end functional groups and molecular weight profiles to the commercially available methoxy-poly(ethylene glycol) (2000 g mol^{-1}), which was used as a comparison in the dendrimer modifications.

Here we have compared polymers with average molecular weights of ~2000 g mol^{-1}, but it is important to note that the number of repeat units is different for each polymer at this same molecular weight. The theoretical number of repeat units for each polymer at this molecular weight decrease in the order PEG > PMOx > PEOx > PEsterOx (n = 44, 23, 20, 14). The use of PEG and POZ at ~2000 g mol^{-1} was largely decided from the literature where PEG has been extensively studied, and comprehensively summarised by Kaminskas, Boyd and Porter.[6] Increasing the molecular weight beyond this would only lead to a reduction in the maximum drug loading (weight %) and make purification by ultrafiltration more difficult. The POZs were all polymerized from freshly distilled monomer, under anhydrous conditions to maintain a living polymerisation and have low polydispersity. Sodium methoxide was used to quench the polymerizations of PMOx and PEOx under conditions designed not to cleave the amide bonds. Loss of the *tert*-butyl ester protecting group at the initiator end after treating with sodium methoxide indicated transesterification had taken place as an unwanted side-reaction. Sodium hydroxide solution was added after quenching to deprotect the newly formed methyl ester. Full conversion of the monomer and proof of the initiation from *tert*-butyl bromoacetate is demonstrated in the ^{1}H NMR spectra of the reaction mixture before quenching (ESI S2 Fig. 17†) showing the *tert*-butyl ester peak at 1.49 ppm and oxazolinium protons at 4.59 ppm and 5.01 ppm, which disappeared after addition of sodium methoxide.

The ester POZ was not quenched with sodium methoxide because the resulting methyl ester at the α-chain end could not be hydrolysed without backbone esters also being cleaved.



Scheme 1 Polyoxazoline synthesis route.

Instead, ethyl thioglycolate was used to quench the chain-end providing an ethyl ester terminal group and preserving the *tert*-butyl ester at the α-chain end. Ultimately the ethyl ester provided another carboxyl group at the terminal chain-end and yet another site for conjugation of coumarin. The synthetic routes to the POZs are summarized in Scheme 1 and the characteristics are summarized in Table 1.

POZ and PEG coupling to G5-PLL dendrimer. Polymers **1a–d** were used to modify the surface of the generation 5 L-lysine dendrimer using the coupling agent 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM) in a random modification *i.e.* some chains may have substituted onto α-amines and others onto ε-amines. For PMOx, PEOx and PEG the modifications were performed in aqueous conditions using DMTMM chloride, which has been reported to be stable in water for several days[23] and a good option in sterically hindered systems for which slow coupling is problematic. The ester POZ (**1d**) had limited water solubility and the coupling had to be performed in DMF with the tetrafluoroborate salt of DMTMM, which is reported to be stable in organic solvent.[24] VivaFlow® cassettes proved to be an efficient way to purify the dendrimers and gave good yields (69–85%). The removal of the excess linear POZ could be followed using HPLC-SEC-UV at 220 nm (example in ESI Fig. 18†). Following purification, the removal of linear material was also confirmed by GPC (ESI Fig. 19† showing an overlay of the linear PMOx and PMOx-modified dendrimer). Previous attempts using dialysis (25 kDa MWCO) for several days did not yield material free of linear POZ or PEG (data not shown). When purified, the POZ coated dendrimers had hydrodynamic diameters in the region of 8–10 nm as measured by DLS and high molecular weights (79.5 kg mol^{-1} & 100.0 kg mol^{-1}) with narrow polydispersities (<1.1). The PEG modified dendrimer (**2c**) was found to be slightly larger at 14 nm, which might be explained by the longer chain length of PEG compared to the POZs (see Table 2). Another explanation could be that there are more bound water molecules to PEG than POZ given the differences in structure. The polydispersity indices of the sub-9 nm particles were not accurately determined due to interference in the intensity signal on the DLS instrument which was not thought to be polymer related. An interesting observation from the DLS data was that the zeta potentials for **2a–c** were almost neutral despite the residual amines from the unmodified lysines of the dendrimer. This suggests that the dendrimer surface was shielded by the presence of the POZ and PEG. However, in the case of the **2d** there was a positive zeta potential measured that then became negative after reacting the amines with succinic anhydride (Table 2). This could be explained by the relatively poor solubility of the polymer driving a compacted conformation as well as a shorter chain length (n = 16) compared to PEG (n = 44) and PMOx (n = 24). The result is that the coverage was likely insufficient to mask the surface charge.

Table 1 Polyethylene glycol and polyoxazoline characteristics

Sample	Yield [%]	DP (theor.)	DP (NMR)	M_n (GPC) kDa	PDI (GPC)
HOOC-PMOx-OMe (**1a**)	89	23	24	2.2	1.09
HOOC-PEOx-OMe (**1b**)	83	20	23	2.1	1.08
HOOC-PEG-OMe[a] (**1c**)	—	45	44	2.1	1.06
HOOC-PEsterOx-COOMe (**1d**)[b]	90	14	16	3.1	1.12

[a] Commercially available PEG (Iris Biotech). [b] Bimodal by aqueous GPC.

Biocompatibility studies. The Cytotoxicity studies were performed in the presence of a fluorescent marker to measure apoptosis. The fluorescence intensities indicated that at the various time points of incubation, the POZ and PEG modified dendrimers were undergoing much less apoptosis that the unmodified dendrimer. For example, at 24 h there was 2–3% of the cells undergoing apopotosis compared to 36% for the unmodified dendrimer at a concentration of 0.5 mg mL^{-1} (mouse endothelial cells NIH-3T3) (Fig. 1). This was largely seen across all of the time points measured, with some small variations at 96 h. The images captured reflected what was measured by the fluorescence, with large numbers of the cells

Table 2 Unmodified and modified dendrimer characteristics

Sample description	Yield [%]	Estimated no. of substituted amines (^{1}H NMR)	M_n (kg mol^{-1}) (GPC)	PDI (GPC)	R_D (nm) (GPC)	R_D (nm) (DLS)[a]	Zeta potential (DLS)
G5-PLL-NH_2	—	—	ND	ND	ND	ND	32.1 ± 5.07
G5-PLL[PMOx]$_x$[NH_2]$_y$ (**2a**)	70	35	79.5	1.06	10.9	9.04	3.2 ± 6.80
G5-PLL[PEOx]$_x$[NH_2]$_y$ (**2b**)	69	38	100.0	1.05	11.8	10.24	2.76 ± 4.26
G5-PLL[PEG]$_x$[NH_2]$_y$ (**2c**)	79	30	80.7	1.09	14.0	14.54	−0.514 ± 3.92
G5-PLL[PEster]$_x$[NH_2]$_y$ (**2d**)	82	28	ND	ND	ND	8.56	10.3 ± 5.41
G5-PLL[PEster]$_x$[COOH]$_y$ (**3d**)	94	64[c]	ND	ND	ND	7.87	−12.1 ± 5.63
G5-PLL[PAcid]$_x$[COOH]$_y$ (**4d**)	89	64[c]	117.2	1.04	12.6	6.73 (8.93[b])	−36 ± 12.2

ND = not determined. [a] Volume average. [b] Measured in GPC eluent (40 : 10 mM $NaNO_3$: NaH_2PO_4 in water). [c] Remaining amines were modified with succinic anhydride.

undergoing apoptosis for the unmodified dendrimer, whilst the cells incubated with the modified dendrimers were similar in appearance to the saline control. The modification of the dendrimer with POZ and PEG had removed and masked some of the cationic charge from the surface of the dendrimer and provided a barrier to membrane disruption, which was consistent with previously reported studies.[27]

Blood compatibility studies were performed on the various modified dendrimers. Firstly, haemolysis experiments were performed, which was a measurement of the haemoglobin (Hb) released by the red blood cells after rupture of the cell. The results showed that the PEG and POZ modified dendrimers were found to be non-haemolytic (ESI Fig. 20†) where the haemoglobin measured was lower than the limit of detection for the instrument (<0.3 g dl^{-1}). Only a small amount of haemolysis (2.45 g dl^{-1}) was observed for the unmodified dendrimer but this was mainly due to the aggregation of the blood cells. The second experiment for blood compatibility was red blood cell aggregation, and was determined though microscopy. The unmodified lysine dendrimer caused severe

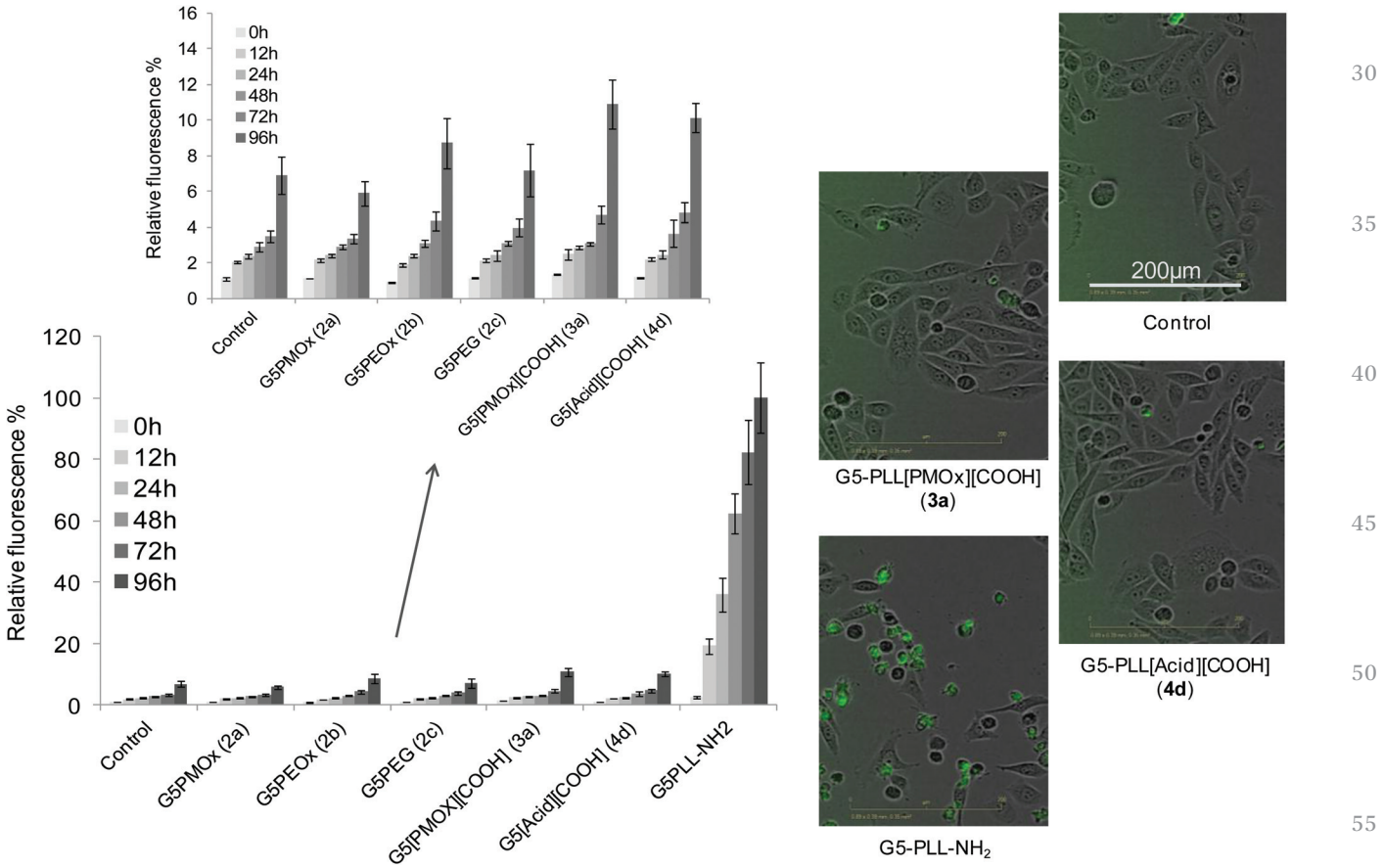


Fig. 1 Cell apoptosis studies. Graph showing relative apoptosis (measured by fluorescence and compared to unmodified dendrimer at 96 h over various time points). Right showing representative images of the cells at 24 h.

aggregation of the red blood cells forming a continous network of red blood cells (Fig. 2). The POZ and PEG modified dendrimers did not cause any observable aggregation of the red blood cells and looked consistent with the saline control.

The concentrations for the blood compatibility and cytotoxicity were chosen to reflect a scenario of an IV administered dendrimer at a reasonable dose of 50 mg kg^{-1} in mouse. The hypothesis being that a mouse weighing 30 g would receive approximately 1.5 mg of polymer per dose and therefore initial concentrations would be highest in plasma followed by dilution and distribution/clearance to give lower final tissue concentrations.

Coumarin loading onto POZ modified dendrimers. A derivative of coumarin with a primary amine attachment point was used as a model compound to conjugate to the dendrimers. This compound offered the benefit of a stable amide bond to the dendrimer to aid with the purification and analysis. The aromatic protons made the quantification of the loading straightforward by ^{1}H NMR since there are no other interfering signals from the dendrimer in this region. Here we wanted to show that by adapting the main-chain functionality of the POZ from a simple alkyl group into a useful moiety (carboxyl groups) there could be significantly more sites available for conjugation and therefore could achieve higher loading. The remaining lysine amines on the POZ modified dendrimers (**2a** and **2d**) were reacted with succinic anhydride to convert them into carboxyl groups (dendrimers **3a** and **3d**). Polymer 3d then underwent a mild methyl ester deprotection step using LiOH in MeOH/H_2O overnight to convert the main-chain esters into carboxyl groups to generate further sites for conjugation of the coumarin (polymer **4d**). Whilst the PMOx modified dendrimer (**3a**) had ~32 theoretical sites on the dendrimer for conjugation of courmarin, the carboxyl-functional POZ dendrimer (**4d**) had ~576 (18× more). However, it is well known in a drug delivery scenario that overloading polymeric carriers with hydrophobic species can lead to solubility problems/aggregation in solution. Therefore, we aimed to modify a conservative 30% of the carboxyl groups (173 sites). The conjugation of coumarin to the dendrimers was performed using DMTMM·BF_4 in anhydrous DMF using a slight excess of the coumarin for polymer **3a** 40 molecules per dendrimer, 1.25 eq.) and the intended 173 molecules per dendrimer for polymer **4d**. The loadings of coumarin were measured using ^{1}H NMR (Fig. 3).

The loadings were found to be 6.4 wt% (25 molecules per dendrimer) for the PMOx modified dendrimer and 31 wt% (166 molecules per dendrimer) for the carboxyl-functionalized dendrimer (6.6× more loading). To determine whether the high coumarin loading on **4d** (resultant polymer **5d**) had induced the formation of aggregates and micellar structures,

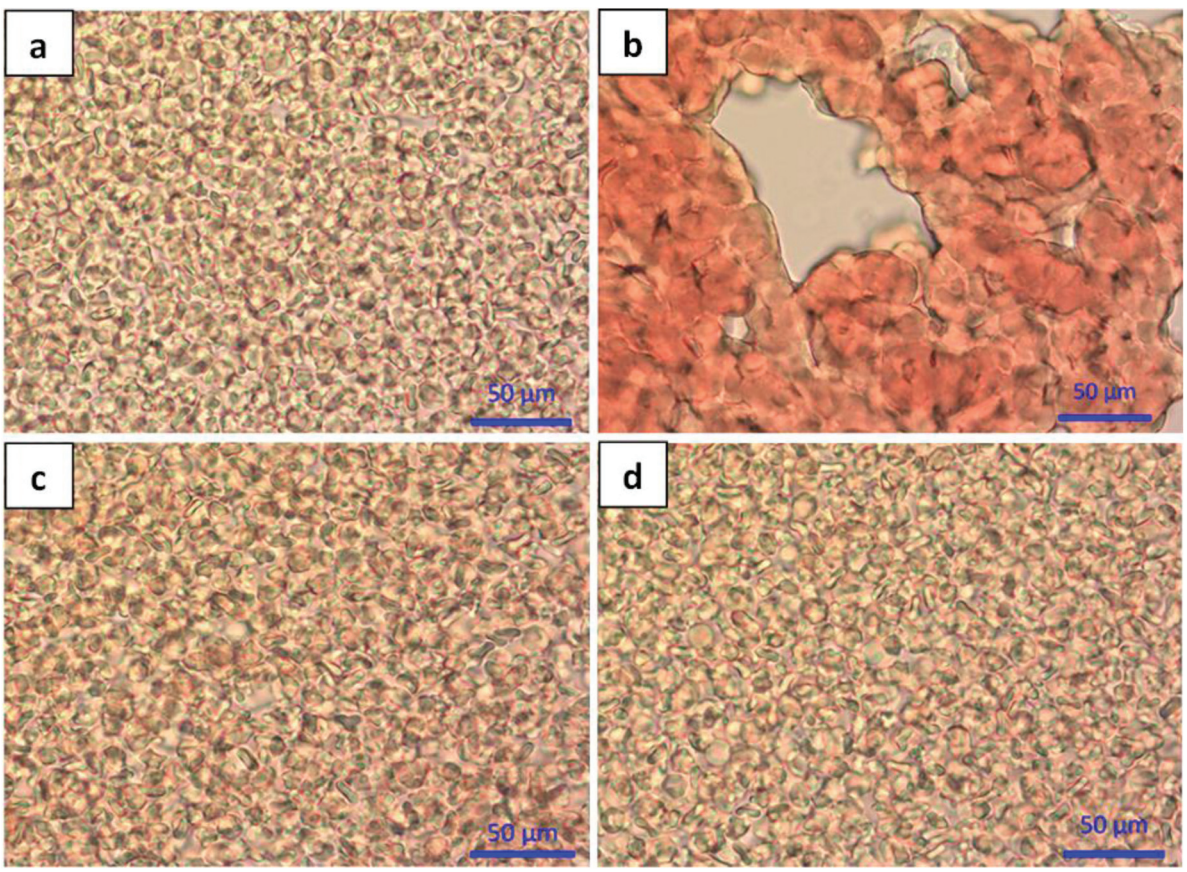


Fig. 2 Blood compatibility aggregation study of unmodified *versus* PEG and PEOx modified dendrimers (a = saline, b = unmodified dendrimer, c = PEG modified and d = PEOx modified).

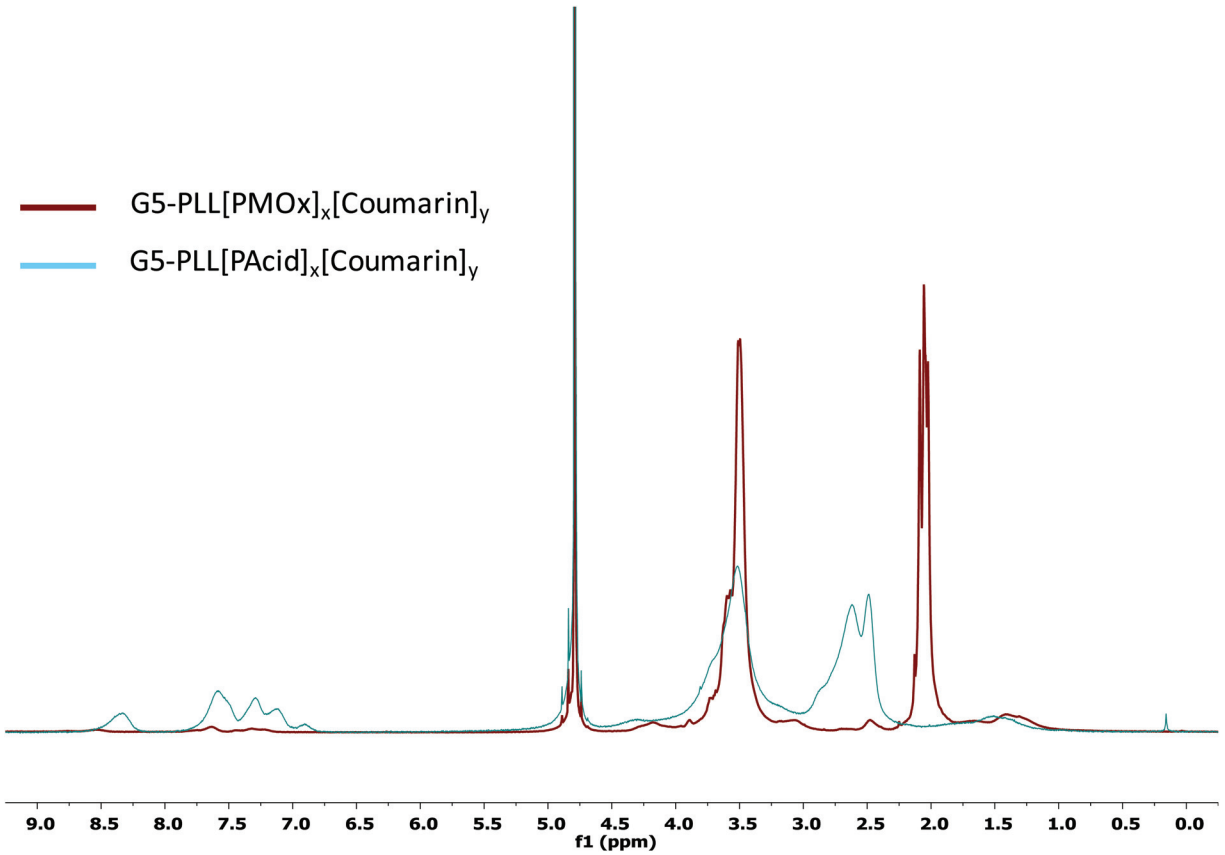


Fig. 3 ^{1}H NMR (400 MHz, D_2O) spectra for the coumarin conjugated POZ dendrimers showing 6.4 wt% loading for PMOx modified dendrimer and 31 wt% loading for the carboxyl functional POZ dendrimer.

DLS was performed. For the polymer conjugate derived from the PMOx modified dendrimer **4a** (25 coumarin molecules) there was no observable change to the size distribution (ESI Fig. 21†). For the coumarin loaded carboxyl-functional dendrimer, **5d** there was a small reduction in hydrodynamic volume accompanied with a tail of larger particle sizes observed in the size distribution when compared to the precursor (ESI Fig. 22†). However, when repeated in a more appropriate buffer (as for GPC) this was not observed (ESI Fig. 23†). The absence of higher molecular weight species was strengthened from the GPC data where no aggregation or larger particles were observed in the light scattering (ESI Fig. 24†).

Clinically, high drug loading on drug delivery systems is desirable. Historically many polymer-drug conjugates have suffered from low drug loading and typically have less than 10% by weight active compound (*e.g.* HPMA-Doxorubicin, PK1 and PEG-Camptothecin, PROTHECAN).[28] Low drug loading drives the need for very potent drug molecules, which are not always possible or desirable. Alternatively, large amounts of the nano-carrier are required often resulting in high number of nanoparticles dosed per injection. Both the quantity of nano-carrier and the number of particles being dosed carry a toxicity risk, either from the material itself or excessively large numbers of nanoparticles. More extensive pre-clinical toxicity studies will be required if a clinically unprecedented concentration of materials is dosed and regulatory hurdles will be higher. Additionally, from a practical perspective, in pre-clinical development where injection volume is limited to 5 mL kg^{-1} in many species, doses may be limited due to solubility or physical stability issues. Similarly in the clinic, low drug loading can result in large volumes or a large number of vials with additional challenges to patient, clinical staff and costs. Many of the polymers used in polymer therapeutics are bespoke and are therefore expensive with often non-standard and extensive characterisation requirements to ensure quality and reproducible *in vivo* performance, thus, minimising quantities from a cost of goods perspective will be desirable. Therefore nano-carriers such as the dendrimers described in this paper with the capacity for considerably higher drug loading and considering the amount required to deliver 100 mg dose (in man) provide a more efficient delivery system and therefore have benefits in terms of development and commercialisation and patient/clinician acceptability.

Conclusions

Polyoxazolines have already shown great promise in the field of polymer therapeutics and we wanted to extend their use to coating materials for L-lysine dendrimers. Three different poly-

oxazolines were synthesised with average molecular weights in the region of 2000 g mol^{-1} to compare with a commercial methoxy-PEG. A generation 5 L-lysine dendrimer was then modified with these chain-end functional POZs and PEG though an amide coupling step. We demonstrated that the PMOx and PEOx coated lysine dendrimers have similar properties to the PEG coated equivalent but with slightly lower hydrodynamic diameters. We imagined that one major benefit of POZ over PEG came from the mainchain functionality that it could offer and therefore a POZ with main-chain ester functionalities was synthesized and used to modify the dendrimer, which were subsequently deprotected to give carboxyl groups. Whilst the PEG and PMOx dendrimers with 50% coating modification were limited to ~32 coumarin molecules per dendrimer, the novel carboxyl functional POZ coating had the potential to hold up to ~576, albeit that a rational choice to prevent aggregation was to conjugate coumarin to only 30% of these (173 molecules). The use of a carboxyl-functional POZ has provided a novel approach for generating large numbers of conjugation sites for drug molecules to improve the loading, whilst other approaches have been limited by the residual surface functional groups from the ultimate generation of the dendrimer itself.

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